Chapter 2

Bloom syndrome & the BLM helicase

Bloom Syndrome

Bloom Syndrome (BS) is a rare autosomal recessive disorder characterized by short stature, immunodeficiency, high incidence of type II diabetes, reduced fertility, UV sensitivity, and a strong cancer predisposition (1). The disease was first described in 1954 (2) and is studied extensively as a model disease for genome instability and cancer development.

BS is caused by mutations in the *BLM* gene, which encodes for the BLM helicase (1). The disease occurs most frequently in individuals of Ashkenazi Jewish decent, due to one particular mutated *BLM* allele (*BLM*^{ASH}) found in this population (3). *BLM*^{ASH} carrier frequency within the Ashkenazi population has been determined at 1/107 and 1/46,000 individuals of Ashkenazi decent have Bloom Syndrome (4). The frequency of mutant *BLM* alleles in the general population is too low to estimate and it is believed most cases are the result of *de novo* mutations (5). Since 1954, more than 300 cases of BS have been reported. Most of these patients are admitted into the BS registry, where patient information, including symptoms and disease progression are stored (6). As of the beginning on 2016, 265 cases of BS have been admitted into the registry (6).

BS is characterized by a wide range of symptoms. The first diagnostic marker is a below average height and weight: the mean birth weight is 1.8 kilograms for both males and females. Proportional short stature remains throughout life, with a mean adult height of 149 cm and 138 cm for males and females, respectively (6). The exact reason for this short stature is not known, but there are two possible explanations that both involve deficiencies in cell division. BS cells display a reduced speed of proliferation *in vitro* (7), as well as increased levels of apoptosis as a result of stalled replication forks (8). During embryonic and foetal development cells divide rapidly and the reduced number of cells in individuals with BS could be due to either the reduced output of cells because of the slow cell division, increased levels of apoptosis, or a combination of both.

Patients start developing respiratory and auditory tract infections during early childhood and blood samples from patients display low levels of plasma globulins, which are characteristic of immunodeficiency (1). Chronic lung disease was diagnosed in 2.6% of patients (6). It has been hypothesized that immunodeficiency in BS patients is due to excessive recombination during immune cell activation (1), but several studies reported no abnormalities in Variable, (Diversity), and Joining gene segment recombination in BS cells (9-11). Interestingly, BLM does appear to play a role in Immunoglobulin (Ig) gene conversion (12). Reduced Ig gene conversion frequency in BS cells could explain the immunodeficiency seen in BS patients. It is also possible that immune cells lacking BLM cannot achieve the high levels of proliferation associated with a normal immune response due to either problems with cell division or increased apoptosis during rapid cell division.

Interestingly, 16% of patients develop diabetes at ages ranging from 4 to 45 years old (6). Although the exact reason is not known, reactive oxygen species (ROS) could be a potential explanation. Bloom Syndrome cells produce higher levels of ROS than normal cells (13,14) and damage caused by ROS has been linked to diabetes (15).

Patients also display a strong sensitivity to UV radiation and develop erythema in parts of the body exposed to sunlight: mainly face, neck, shoulders, arms, and hands (1). This corresponds with a UV sensitivity seen in BS cells, in which UV

radiation induces high levels of apoptosis (16) and reflects a DNA repair defect caused by the absence of BLM.
Quite strikingly, individuals with BS show marked subfertility. Successful pregnancies in women are rare but have been reported (17,18), but males are sterile due to the absence of functional spermatozoa in their sperm (19). BLM and its homologs in yeast and mouse have been shown to play a role in meiotic recombination (20-23), and a deficit in proper meiotic recombination is a likely reason for the reduced fertility. Indeed, BLM is required for normal spermatogenesis (22), even though females can apparently still produce healthy oocytes. The main difference between male and female meiosis is the presence of XY-bodies, which pair the non-homologous X- and Y-chromosomes in male cells (24). Meiotic recombination between the X- and Y-chromosomes is limited to pseudoautosomal regions and no recombination should occur outside of these regions (25). BLM appears to specifically prevent recombination between non-homologous chromosomes (22) and perhaps also between non-homologous regions on the X- and Y-chromosomes. This could explain the critical role BLM plays in male, but not female, meisosis.
Arguably the most serious symptom of the disease is a strong predisposition towards a wide range of cancers, which includes leukemia's, lymphomas, carcinomas, and rare tumours (1). Interestingly, leukemia seems to be the predominant type of cancer in children, lymphomas are found in both children and adults, while carcinomas start to appear during adolescence and become the predominant cancer type in adulthood (1). Out of the 265 individuals in the Bloom Syndrome registry, 122 (46%) have so far been diagnosed with cancer (6). Roughly one third of patients suffering from cancer will develop at least one secondary malignancy at the same time or later in life (1). These later tumours can be of the same or a different origin than the primary tumour. For example, one patient was diagnosed with basal cell carcinoma 4 times within 5 years, while another developed separate instances of lymphoma, left breast carcinoma, colon carcinoma, and right breast carcinoma over the course of 17 years (1). In general, time between diagnosis of first and second cancers has varied between 9 months and 13 years (1). The mean age at death of BS patients is 26 years (6).
BS cells are characterized by marked genome instability. The cells display a high sister chromatid exchange (SCE) rate (26), an elevated spontaneous mutation rate (27,28), a high frequency of micronuclei (29-31), increased DNA breaks during S-phase (32), high levels of replication intermediates (33), delayed DNA chain growth during replication (7), and an increased sensitivity to DNA damaging agents such as mitomycin C (34), ethyl methanesulfonate (35), hydroxyurea (36), formaldehyde (37), UV radiation (16), X-radiation (38), and gamma radiation (39).

BLM

The BLM gene was identified as the causative gene for Bloom syndrome in 1995 (40), after it was shown that the BS phenotype can be corrected by the introduction of normal human chromosome 15 into BS cells (41). The causative mutation was subsequently mapped to 15q26.1 (42). The BLM gene encodes for a 1417 amino acid protein belonging to the family of RecQ helicases (40). The high SCE rate in BS cells could be rescued by somatic intragenic recombination in cells containing heterozygous BLM mutations (43) and by transfection of normal *BLM* cDNA into BS cells (44), further proving that mutations in BLM are the cause of BS.

BLM is a RecQ helicase

The RecQ helicases are highly conserved from bacteria to humans and play important roles in the maintenance of genome stability (45). The *E. coli* RecQ helicase is the founding member of this family of helicases and homologs have been identified in several strains of yeast (46,46,46,47) and in higher eukaryotes, including *Drospohila melanogaster* (48,49), *C. elegans* (50), and *Mus musculus* (51). Bacteria and yeast have one RecQ helicase each, while humans have five known member RecQ helicases: RECQ1, WRN (also called RECQ2), BLM (also called RECQ3), RECQ4, and RECQ5 (Figure 1). The RecQ helicases contain three conversed domains: the helicase/ATPase domain (which consist of the DEAD/DEAH box domain and a C-terminal helicase domain), the RecQ carboxy-terminal (RQC) domain, and the Helicase and RNAse D C-terminal (HRDC) domain (45) (Figure 1). The RQC domain is found solely in the RecQ helicases, although it was most likely lost during evolution of RECQ4 and RECQ5, and has been linked to dsDNA binding (52). On the other hand, the HRDC domain seems to confer a DNA binding substrate specificity (53,54).

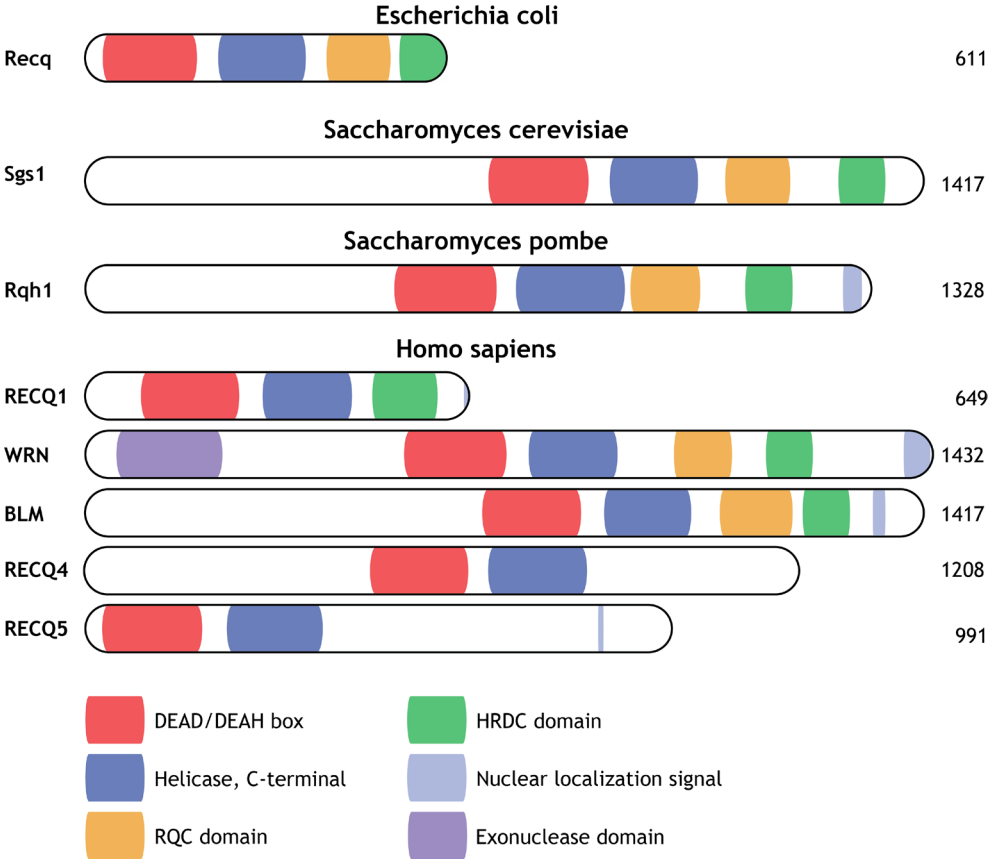

Figure 1 | RecQ helicases. Representation of the RecQ helicases found in bacteria, yeast, and human. All members contain the DEAD/DEAH box (shown in red) and the C-terminal helicase domain (dark blue). Also depicted are the RQC (yellow) and the HRDC (green) domains, nuclear localization signals (light blue) found in several family members and the exonuclease domain (purple), which is only present in the WRN protein. Number of amino acids is displayed on the right.

Mutations in several RecQ helicases cause diseases associated with severe genomic instability (Table 1): Bloom syndrome (BLM) (1), Werner syndrome (WRN) (55), Rothmund-Thompson syndrome (RECQ4) (56), RAPADILINO syndrome (RECQ4) (57), and Baller-Gerold syndrome (RECQ4) (58). Whether these latter three diseases are truly separate entities or if each lies within the spectrum of one overlapping disorder is the subject of ongoing debate (59). Mutations in RECQ1 are associated with a predisposing to breast cancer (60,61), but do not appear to result in a genetic disorder. It is unknown whether mutations in *RECQ5* do not result in a phenotype, or if such mutations would confer embryonic lethality. Quite strikingly, each of the RecQ syndromes is associated with growth retardation and cancer predisposition, although the exact symptoms and types of cancer vary between the diseases (Table 1). Thus, the cancer predisposition seen in each of the syndromes highlights the role RECQ helicases play in the maintenance of genome stability.

Disease	Causative gene	Symptoms	Cancer predisposition	Ref
Bloom syndrome	BLM	Short stature, immunodeficiency, UV sensitivity, type II diabetes, reduced fertility	Leukemia, lymphoma, carcinoma, solid tumours	(1)
Werner syndrome	WRN	Greying of the hair, cataracts, osteoporosis, atherosclerosis, type II diabetes	Sarcoma, rare tumours	(55)
Rothmund-Thompson syndrome	RECQ4	Bone malformations, alopecia, tooth and nail dystrophy, cataracts, abnormal skin pigmentation	Carcinoma, (osteo)sarcoma	(56)
RAPADILINO syndrome	RECQ4	Short stature, radial ray and patellar malformations, arched or cleft palate, dislocated joints	Osteosarcoma, lymphoma	(57)
Baller-Gerold syndrome	RECQ4	Premature fusion of skull bones, radial ray malformations, growth retardation	Osteosarcoma, lymphoma, melanoma	(58)

Table 1 | RECQ syndromes. Overview of the different syndromes associated with mutations in RECQ helicases. For each syndrome, the mutated helicase, most common symptoms, and types of cancer predisposition are shown.

BLM helicase action and substrate specificity

BLM is a 3' to 5' DNA helicase (62) with a high specificity for certain secondary structures in the DNA, such as replication forks (63), D-loops (64), Holliday junctions (65), and guanine-quadruplex (G4) structures (66,67) (Figure 2). D-loops are structures where a DNA duplex is invaded by a third (homologous) strand, which pairs with one of the duplex strands and displaces the complementary strand (Figure 2B). D-loops occur mainly during DNA double-strand break (DSB) repair by homologous recombination (HR) (68) and at the ends of telomeres, where the 3' overhang folds back and invades the DNA helix to form a T-loop (69). The Holliday junction (HJ) is a structure that contains four DNA strands annealed together (Fig-

ure 2C). Holliday junctions are recombination intermediates that occur during DNA replication and HR (70). G4s are secondary structures formed by specific G-rich motifs (71). The consensus sequence for a G4 motif contains four stretches of at least three guanine residues (Figure 2D). These residues can form a stacked planar structure which is stabilized by the presence of Hoogsteen bonds between the G-residues. G4 structures occur mainly within a single DNA strand, but evidence exists for inter-strand G4 structures forming between DNA strands and DNA:RNA hybrids as well (71).

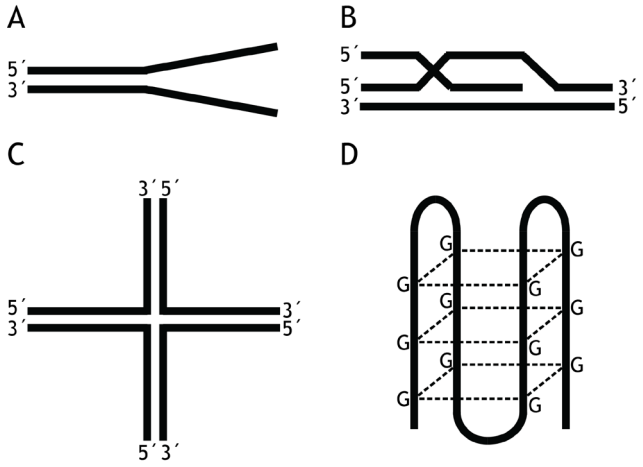

Figure 2| Substrates for BLM helicase action. BLM displays a high reactivity for a number of secondary structures. It is capable of regressing replication forks (A) and unwinding D-loops and R-loops (B; invading strand can be DNA (D-loop) or RNA (R-loop)), Holliday junctions (C), and G quadruplexes (D).

Regulation of BLM expression and localization

The BLM protein is expressed ubiquitously and the highest protein levels are found in tissues displaying high fractions of proliferative cells, such as lymphoid tissues and most tumours (72). BLM expression increases sharply during S-phase of the cell cycle and peaks in G2/M-phase, before decreasing in the subsequent G1-phase after mitosis (73-75). BLM contains a strong nuclear localization signal and is found solely in the nucleus (76), where it co-localizes with the promyelocytic leukemia (PML) protein to PML nuclear bodies (29). PML bodies contain several DNA repair proteins, including RAD50, RAD51, RAD52, MRE11, NBS1, TOP3α, and p53 (77). It is believed that these proteins are sequestered to PML bodies in undamaged cells to prevent aberrant DNA metabolism (78). Indeed, *PML*$^{-/-}$ mouse cells lose BLM localization to PML bodies and display elevated SCE rates (78).

BLM is subjected to different post-translational modifications during the cell cycle and in response to DNA damage (77). BLM is constitutively phosphorylated by CHK1 at residue S502 to prevent its degradation by cullin-3 (79). BLM is also phosphorylated during S-phase at residue S338 and this modification is believed to stabilize BLM protein levels (80,81), although the exact phosphorylation site and stabilizing mechanism are under debate. One study reports that BLM is phosphorylated at S338 and that this modification enhances an interaction with TopBP1 to prevent BLM degradation (80). Another study however reported that the BLM-TopBP1 interaction depends on phosphorylation of S304 and that this interaction is not required for the stabilization of BLM (81). In either case, BLM expression increases during S-phase, which is consistent with its role in DNA replication and recombination (as discussed in later sections).

BLM is further phosphorylated during mitosis. Phosphorylation of residues S714 and T766 is mediated through CDK1 and this is believed to exclude BLM from mitotic chromatin and prevent interference with chromosome condensation (82). BLM is also phosphorylated at S144 by MPS1 and may subsequently play a role in the mitotic spindle checkpoint (83). After mitosis and entry into G1, BLM is ubiquitinated by MIB at K38, K39, and K40 and targeted to the 26S proteasome for degradation (80), leading to the observed decrease in protein levels.
BLM expression increases after induction of DSBs by X-radiation or etoposide and the protein aggregates in distinct foci at the sites of the DSBs (84). Furthermore, BLM is phosphorylated at T99 and T122 by ATM and ATR in response to DNA damage, resulting in dissociation from PML bodies and localization to the sites of DNA damage (8,35,74). BLM is SUMOylated in response to replication stress, leading to BLM localization at the sites of stalled replication forks (85,86), where BLM can aid in replication restart (87) (see next section).

BLM prevents replication fork collapse and double-strand break formation
Smooth DNA replication is of critical importance for proper transmission of genetic material to daughter cells and to prevent mutations from occurring during cell division (88). Faithfull replication depends on multiple processes including proper unwinding of the DNA duplex to create single-strand DNA (ssDNA) that can be used as a template for replication, and the repair or resolution of any barriers that might stall the replication fork. When the replication fork encounters a barrier, such as single-stranded nicks, lesions, or secondary structures (like G4s) the fork will stall and replication cannot continue until the barrier is overcome (88). The cell has multiple pathways to resume DNA replication after a fork stalls and BLM plays a crucial role in several of these pathways (89).
The first indication that DNA replication might be impaired in BS cells came in 1975, when it was reported that the speed of replication fork progression was lower in BS cells than in normal cells (7). Although the exact cause of this phenotype is not known, it could be due to delayed processing of Okazaki fragments into a continuous lagging strand. BLM has been shown to promote Okazaki fragment processing by directly interacting with and stimulating the activity of FEN-1, the endonuclease required for removing overhangs from Okazaki fragments (90,91). BS cells also display impaired activity of DNA ligase I, the major DNA ligase in proliferating human cells (92-94). BS cells have normal amounts but reduced activity of DNA ligase I, suggesting that BLM stimulates DNA ligase I activity (94). BS cells also display abnormal replication intermediates (33,95) and it was later shown that BLM localizes to the sites of stalled replication forks (87,96) where it recruits other proteins involved in stalled replication fork signalling and restart, including the MRE11/RAD50/NBS1 (MRN) complex (87,97) and p53 (98).
Upon recognition of a stalled fork, it can be restarted using several different pathways: single-strand annealing, template switching, and HR-mediated repair (see also Chapter 1, Figure 1). The single-strand annealing pathway and the template switching pathway are initiated by regression of the replication fork, a process which is stimulated by the presence of BLM (63,99,100). The ssDNA generated by end-resection can be extended and re-annealed, after which the fork can be reversed and replication restarted. Both ssDNA annealing and fork reversal are stimulated by BLM (101). Alternatively, a D-loop can be formed where one na-

scent DNA strand is used as a template for replication of the other nascent strand. Template switching results in the formation of a double Holliday junction (dHJ), dissolved by BLM (102), allowing replication to continue. If replication fork restart by single-strand annealing or template switching is not possible, the fork will collapse and be removed from the DNA. This results in a one-ended DSB, which is then repaired via HR. This process does not rely on BLM and results in complete restart of replication and leads to SCE formation (89,103). This reliance on HR-mediated fork restart in BLM deficient cells can (partially) explain the high SCE rates seen in these cells.

Clearly BLM plays an important role in DNA replication, and is specifically required to prevent replication fork collapse and the formation of DSBs. It is also possible that BLM plays a role in removing barriers for replication ahead of the replication fork. For example, G4s would pose a strong barrier for replication and could lead to stalled forks if not removed. BLM has been shown to unwind G4 structures *in vitro* (66,67,104) and could potentially also prevent fork stalling by removing replication barriers (105). Such a mechanism has so far not been confirmed for BLM or its homologs *in vivo*, but was reported for two other helicases that can unwind G4s: yeast Pif1 (106) and mammalian FANCJ (107).

BLM prevents cross-overs during DNA repair by homologous recombination

Double strand breaks are the most deleterious form of DNA damage, and one unrepaired DSB is sufficient to prevent a cell from dividing (70). The two major pathways for DBS repair in mammalian cells are non-homologous end joining (NHEJ) and HR (108) (see also Chapter 1, Figure 3). NHEJ is the preferred pathway during G1 phase and is used to ligate the ends of broken chromosomes back together. As such, it is an error prone repair pathway and often leads to small insertions or deletions (109). By contrast, HR is active during S/G2 phase and uses an intact homologous template to repair DSBs. HR is generally considered to be an error-free repair pathway (110), although it can lead to mutations and loss of heterozygosity (LOH) (70). The homologous template used for repair is typically a sister chromatid, but the homologous chromosome or even a homologous region on a different chromosome can also be used. This occurs mainly for regions containing a high number of repeats, such as telomeres (111) and ribosomal DNA (rDNA) loci (112).

One of the first signals originating from a DSB is the presence of γ-H2Ax (113). BLM accumulates at the site of DSBs and co-localizes with γ-H2Ax (114,115). Once a DSB is recognized, repair by HR can be initiated by resecting the 5' ends of the break by EXO1 and DNA2 to create 3' overhangs on either end of the break (116). This end-resection promotes repair via HR and inhibits end-joining during S/G2 phase (117). BLM promotes end-resection by EXO1 (118,119) and DNA2 (120), thus promoting repair by HR and ensuring proper and faithful strand invasion and repair. The 3' overhangs will be used to find and invade a homologous sequence, typically the identical location on the sister chromatid. One overhang will invade this homologous sequence and displace one of the DNA strands, leading to the formation of a D-loop (110). At this point the invading strand can be displaced by BLM and used to fill in the other broken strand via a mechanism called synthesis-dependant strand annealing (SDSA) (64). This process ensures that both sister chromatids remain intact and does not result in any cross-over events (70). Alternatively, repair can continue via the double-strand break repair (DSBR) pathway. The second broken

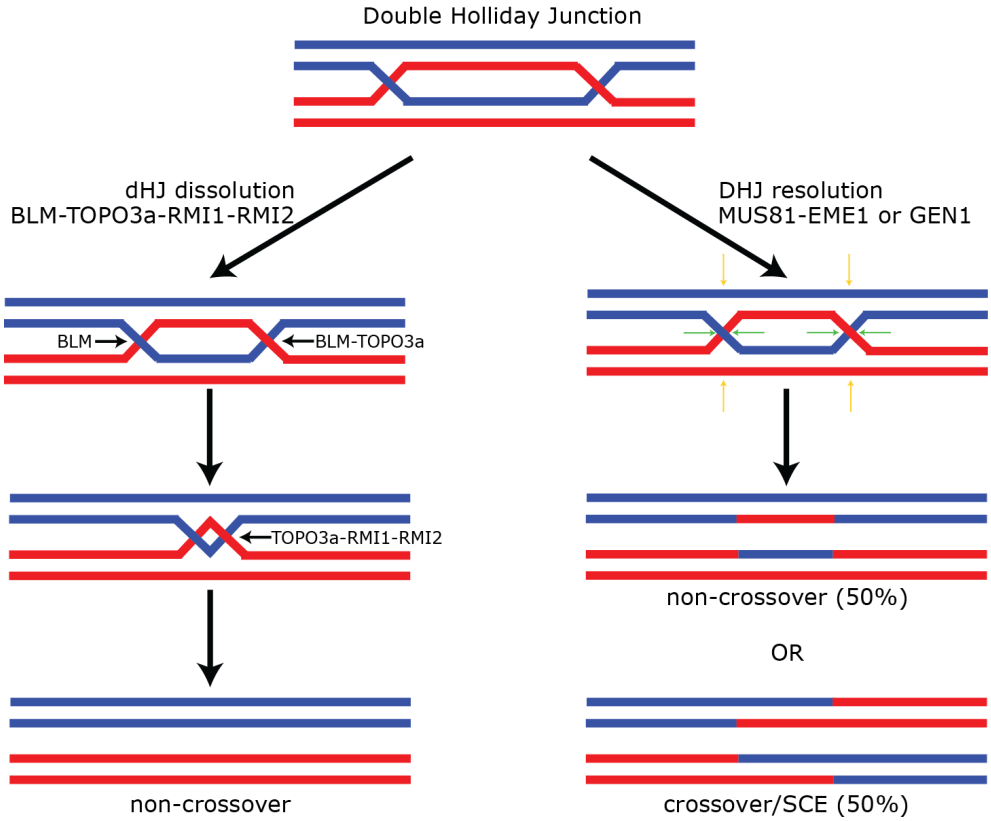

Figure 3| Double Holliday junction processing. Double Holliday junctions are four-way structures that can form during replication fork restart and homologous recombination. BLM, together with TOPO3a, RMI1, and RMI2 is required to properly dissolve these dHJ structures. During dHJ dissolution, BLM and TOPO3a stimulate branch migration of invading DNA strands, resulting in a catenate that is resolved by TOPO3a, RMI1, and RMI2, resulting in proper re-localization of all DNA strands and dHJ processing without cross-overs. In absence of BLM, or if a dHJ cannot be dissolved, dHJ resolution acts as a back-up mechanism. During dHJ resolution, DNA strands are cleaved by MUS81-EME1 or GEN1 and the broken strands are re-ligated on the separate sister chromatids. However, this process can result in either a crossover or a non-crossover event.

strand can be captured by the displaced strand from the sister chromatid and extended using this displaced strand as a template. This will lead to the formation of a double Holliday junction (dHJ) (70). This dHJ can subsequently be dissolved by the BLM-TOPOIIIα-RMI1-RMI2 (BTRR) complex (65,121-123), leading to separation of the sister chromatids without crossing over (Figure 3). If the dHJ cannot be dissolved (for instance because BLM is absent), it is cleaved by the endonuclease complex MUS81/EME1 (124) or by GEN1 (125) in a process called dHJ resolution, which is estimated to result in 50% crossovers (SCEs) and 50% non-crossovers (126). It is clear that BLM plays a critical role in proper repair of DSBs. Stimulation of end-resection by BLM leads to a longer 3' overhang, which promotes proper homology search and strand invasion. In case of insufficient end-resection DSBs are typically repaired via the NHEJ pathway (108). BLM also promotes SDSA by displacing D-loops before a dHJ is formed (64). When SDSA does not occur and the D-loop forms

a dHJ, BLM promotes dHJ dissolution to prevent SCEs. Therefore the absence of BLM could lead to a strong increase in crossovers during HR, and this high SCE rate is exactly what is seen in cells from Bloom Syndrome patients (26).
Although an SCE normally does not include any mutations in the DNA sequence and can be considered error-free, unequal SCEs can occur when the DSB is repaired using a region other than the homologous region on the sister chromatid. These unequal SCEs can lead to loss or gain of DNA sequences (112). If the homologous chromosome is used for repair instead of the sister chromatid a crossover would result in an interhomolog exchange and LOH (127). This can result in heterozygous mutations (e.g. in tumour suppressors or oncogenes) to become homozygous and is a well-known driver of cancer. As would be predicted, loss of BLM has been linked to elevated levels of LOH (128,129). Therefore, the main role of BLM in maintaining genome stability appears to be in the prevention of mutations by unequal SCEs and interhomolog exchanges.

BLM and telomere maintenance
Telomeres are specialized structures present at the ends of linear chromosomes. In vertebrates, a telomere consist of several kilobases of TTAGGG repeats that end in a 3' overhang (130), and the proteins associated with the telomeric repeats (collectively called the shelterin proteins) (131). Telomeres become shorter with each cell division due to incomplete replication at their ends and when they become critically short, the cell cycle is halted and cells become senescent or go into apoptosis (130). If cells escape from this telomere checkpoint, chromosomes with uncapped ends emerge, thus triggering genome instability. Telomeres are thought to have evolved partially as a tumour suppressor mechanism, by limiting the number of divisions (somatic) cells can undergo and thus preventing accumulation of deleterious mutations (130).
Telomeres also prevents the ends of chromosomes from being recognized as DSBs by folding back onto themselves into a structure called a T-loop, where the 3' overhang at the end of the telomere will invade a region of double-stranded telomeric DNA, forming a D-loop. This structure is then stabilized by the presence of the shelterin proteins, effectively hiding the end of the chromosome from the DNA repair machinery (69). Irrespective of whether the very ends of telomeres are hidden by the T-loop or by shelterin-mediated chromosome condensation (132), loss of telomeres can lead to end-to-end fusions. These fused chromosomes will likely break during mitosis, leading to gains or losses of genetic information and genome instability (133).
Replication of telomeres could pose a problem due to the presence of the T-loops and D-loops and the G-rich nature of telomeric repeats, which form the canonical G4 motif. Since BLM can unwind both D-loops and G4s, it might play a role in telomere replication. Indeed, BLM appears to localize to telomeres (134,135) and interacts directly with shelterin proteins TRF1 (136), TRF2 (137), and POT1 (138). TRF2 stimulates BLM to unwind short duplexes *in vitro* (137). BLM is also stimulated by POT1, which binds specifically to telomeric ssDNA and could therefore allow BLM to unwind longer stretches of telomere repeats (138). Unwinding DNA is critical for DNA replication and BLM deficiency causes a decrease in the speed of replication (139). Interestingly, this effect was seen only in the G-rich leading strand, which contains the G4 motifs. Indeed, fork progression was slowed further

by the presence of the G4 stabilizer PhenDC3 (139). Furthermore, BLM deficiency leads to increased numbers of telomere defects, accelerated telomere shortening, and activation of the DNA damage response at telomeres (135).
Because telomeres limit the number of times a cell can divide, tumour cells must somehow overcome this barrier to achieve unrestricted growth. Most tumours do this by upregulating expression of telomerase, the reverse transcriptase used to add telomere repeats to the ends of telomeres (140). However, ~10% of tumours are telomerase negative and maintain telomere length through a recombination based mechanism called alternative lengthening of telomeres (ALT) (141). BLM also localizes to telomeres in ALT cells and BLM overexpression leads to increase in telomeric DNA (142). Interestingly, the BLM homolog Sgs1 is required for ALT in yeast, while *Sgs1*$^{-/-}$ yeast expressing BLM are capable of ALT (143). BLM deficient mammalian cells can probably still perform ALT (144), possibly due to redundancy between the RECQ helicases, and potentially other helicases.
The exact mechanism of ALT is currently unknown, but two models propose that telomeres are elongated either through unequal SCEs between telomeres, or via break-induced DNA replication (BIR), a recombination-dependent DNA replication where a long telomeres serve as a template for replication of shorter telomeres (141). Given the importance of BLM in ALT and that one of its main function is to prevent SCEs, the unequal SCE model does not appear very likely to be true. On the other hand, replication of telomeres using another telomere as a template would involve the formation of D-loops, and potentially double Holliday junctions, that need to be resolved at some point. Given the fact that BLM can dissolve both of these structures, it seems possible that BLM can promote ALT via this recombination-based replication mechanism.

BLM unwinds ultra-fine anaphase bridges to ensure faithful chromosome segregation

Although the role BLM plays in DNA replication and HR are well known, it was recently discovered that it is also required for proper chromosome segregation during mitosis and possibly meiosis. BLM localizes to specific classes of anaphase bridges between sister chromatids which were dubbed ultra-fine bridges, or UFBs (145). These UFBs do not contain histones and cannot be stained using the DNA dye 4',6-diamidino-2-phenylindole (DAPI) (145). They are however coated with several proteins, including PICH, RIF1, and BLM, and can be visualized by staining for these proteins (146). So far three types of UFBs have been identified: the centromeric ultra-fine bridge (C-UFB), the fragile site ultra-fine bridge (FS-UFB), and the telomeric ultra-fine bridge (T-UFB) (146).
Theoretically, UFBs persisting beyond anaphase could lead to improper chromosome segregation and result in aneuploidy and formation of micronuclei containing chromosome fragments lagging behind during chromosome segregation. Indeed, UFBs appear to persist in BLM deficient cells (145) and these cells have previously been shown to contain higher numbers of micronuclei than normal cells (29). Micronucleation and resulting aneuploidy are known drivers of cancer and increased occurrence of aneuploidy could contribute to the cancer predisposition associated with Bloom Syndrome.

BLM can affect gene expression by unwinding G-quadruplexes

G4 structures do not only pose a barrier for replication, but can also play a role in transcription (71). G4 motifs occur frequently in promoters and are believed to influence gene expression, possibly by enhancing or blocking of transcription factor binding (71). Like in replication, G4s can also form a barrier for the progression of transcription machinery, either when they form in dsDNA in front of transcription or in ssDNA formed during transcription. Since BLM can unwind G4s, it seems plausible that it can affect gene expression through this G4 unwinding activity. Indeed, BLM deficient cells show a different gene expression profile than normal cells. One study reported that genes which are upregulated in BS cells are enriched for G4 motifs, both within the gene but also in promoter regions, introns, and downstream of transcription termination sites (147). Another study has linked both up- and downregulation of genes in BS cells and BLM knockdown cells to the presence of G4 motifs in promotors and in the first 250 base pairs of the first exon (148).

Besides the interplay between BLM and G4 motifs, changes in gene expression in BS cells were also linked to the physical interaction between BLM and p53 (149). Absence of BLM leads to a decrease of p53-mediated expression of several genes involved in the cell cycle (149). Reduced expression of these genes could explain the delayed cell proliferation observed in BS cells and some of the symptoms associated with the disease. Interestingly, the *TP53* gene, which encodes for p53, contains G4 structures that regulate mRNA splicing and affects which isoforms of p53 are expressed (150). It is possible that BLM can unwind the G4s in *TP53* and that absence of BLM leads to expression of different p53 isoforms, which could in turn affect expression of genes downstream of p53.

BLM also appears to play a role in preventing transcription-induced DNA damage by unwinding of R-loops. R-loops are DNA:RNA hybrid structures that occur mainly during transcription (151). They occur when nascent RNA molecules hybridize to the complementary DNA strand, displacing the second DNA strand and causing it to loop out (151). If not resolved, an R-loop could block further transcription and affect mRNA expression levels. Persistent R-loops could also pose a barrier for DNA replication and lead to replication fork stalling (151). Interestingly, R-loops closely resemble D-loops, with the major difference that the invading strand is an RNA molecule instead of a DNA molecule (152). Indeed, there are indications that BLM can unwind R-loops (153-155) and it is possible that BLM prevents R-loop induced mutations by preventing their persistence.

BLM is an anti-recombinase

The BLM helicase has been found to play a role in many different cellular processes, ranging from DNA replication and repair to chromosome segregation and transcription (summarized in table 2). When examining the exact roles BLM plays within these processes, a picture emerges of BLM as an anti-recombinase that prevents unwanted exchanges of genetic material between chromosomes. These kinds of exchanges occur predominantly as a result of DSBs and BLM appears to play an important role in preventing the occurrence of such lesions during DNA replication and transcription.

In a normal cell some DSBs will result in a sister chromatid exchange, or even an interhomolog exchange. The frequency of DSBs resulting in exchanges is not known, but is considered to be rather low (70). It seems plausible that the relative

Process	Function	Mechanism	Ref(s)
DNA replication	Lagging strand synthesis	Stimulating FEN-1 activity	(90,91)
	Prevent fork stalling	Unwinding G-quadruplexes	(66,67,104)
	Recruit MRN complex to stalled replication forks	Signal from sites of stalled replication forks	(87)
	Restart replication fork by single strand annealing or template switching	Stalled fork regression	(99,100)
	Restart replication fork by single strand annealing	ssDNA annealing	(101)
	Restart replication fork by single strand annealing	Fork reversal	(101)
	Restart replication fork by template switching	Template switching	(102)
	Restart replication fork by template switching	Double Holliday junction resolution	(102)
	Inhibit HR-mediated repair, SCE formation	Prevent replication fork collapse	(88)
Homologous recombination	Signal from DSB to recruit DNA repair machinery	Facilitate γH2Ax phosphorylation	(114)
	Promote repair by HR, inhibit NHEJ	Stimulate end-resection by EXO1 and DNA2	(116,118)
	Promote homology search, strand invasion	Stimulate end-resection by EXO1 and DNA2	(116,118)
	Promote SDSA, inhibit DSBR	Resolve D-loops	(64)
	Prevent dHJ dissolution and crossover formation	Resolve double Holliday junctions	(121,122)
Chromosome segregation	Promote sister chromatid segregation	Resolve ultrafine bridges	(145,146)
	Prevent micronucleation, aneuploidy	Resolve ultrafine bridges	(145,146)
Transcription	Induce/inhibit gene expression	Unwind G-quadruplexes	(66,67,104)
	Induce gene expression	Stimulate p53 activity	(149)
	Prevent stalling of transcription	Unwind R-loops	(154,155)

Table 2| Summary of BLMs functions in DNA replication, homologous recombination, chromosome segregation, and transcription.

frequency of exchange events is somewhat fixed and that an increase in the number of DSBs will result in an increased number of SCEs. This is supported by experiments where DSBs were induced by treating cells with mytomycin C, resulting in an increase in SCE rates (156). Similar results were obtained by treating cells with low levels of aphidicolin, which induces replication stress through polymerase inhibition and leads to DSBs specifically at fragile sites (157). Breakage at fragile sites is accompanied by high levels of SCEs (158). Altogether this suggests BLM mainly reduces SCE rates by preventing DSBs from occurring. However, BLM also prevents DSBs to be repaired with an SCE outcome. It does so by pushing the repair reaction into the HR pathway and preventing cross-overs from occurring by dissolving dHJs.

When these different functions of BLM are considered, a picture emerges of a helicase that evolved specifically to prevent the occurrence of chromosomal crossovers during the cell cycle. This function of BLM would be especially important in preventing recombination between homologs and the resulting LOH. Indeed, several studies have reported increased LOH in the absence of BLM (159,160). During meiosis, where recombination between homologs is desired, BLM most likely supresses aberrant recombination that would result in unwanted genomic rearrangements.

The role that BLM appears to play in chromosome segregation and specifically the resolution of late replicating regions seems to be separate from its functions in preventing chromosomal crossovers. However, it does seem likely that BLM is required to prevent missegregation of chromosomes and the resulting formation of micronuclei and aneuploidy. Whether this function of BLM has evolved separately from its other functions or if it is a by-product of its role in crossover prevention is currently unknown.

Although the BLM helicase and the phenotype of BS cells have been studies extensively, the exact role of the BLM helicase in maintaining genome stability is incompletely understood. In the next two chapters, we present studies into the role and function of BLM in maintaining genome stability, which should contribute to our understanding of BLM and the genome instability that occurs in its absence.

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